

Microorganisms in the Stratosphere (MIST)
In-Flight Sterilization with UVC LEDs

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Majors: Biology; Molecular Biology & Biochemistry

OSSI/NIFS Summer Session

Date: 29 July 2014

Microorganisms in the Stratosphere (MIST) In-Flight Sterilization with UVC LEDs

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The stratosphere (10 km to 50 km above sea level) is a unique place on Earth for astrobiological studies of microbes in extreme environments due to the combination of harsh conditions (high ultraviolet radiation, low pressure, desiccation, and low temperatures). Microorganisms in the Stratosphere (MIST) will attempt to characterize the diversity of microbes at these altitudes using a balloon collection device on a meteorological weather balloon. A major challenge of such an aerobiology study is the potential for ground contamination that makes it difficult to distinguish between collected microbes and contaminants. One solution is to use germicidal ultraviolet light emitting diodes (UV LEDs) to sterilize the collection strip. To use this solution, an optimal spatial arrangement of the lights had to be determined to ensure the greatest chance of complete sterilization within the 30 to 60 minute time of balloon ascent. A novel, 3D-printed test stand was developed to experimentally determine viable *Bacillus pumilus* SAFR-032 spore reduction after exposure to ultraviolet radiation at various times, angles, and distances. Taken together, the experimental simulations suggested that the UV LEDs on the MIST flight hardware should be active for at least 15 minutes and mounted within 4 cm of the illuminated surface at any angle to achieve optimal sterilization. These findings will aid in the production of the balloon collection device to ensure pristine stratospheric microbial samples are collected. Flight hardware capable of in-flight self-sterilization will enable future life detection missions to minimize both forward contamination and false positives.

Nomenclature

DI	=	deionized
LED	=	light emitting diode
MIST	=	Microorganisms in the Stratosphere
MPN	=	most probable number
UV	=	ultraviolet (400-10 nm wavelength)
UVC	=	ultraviolet C (280-100 nm wavelength)

I. Background/Introduction

Microbial life has been found in nearly every explored environment on Earth. The diversity of microorganisms and their environments suggests that life is able to adapt to almost anything over evolutionary time. This potentially holds true for life that is able to survive in the upper atmosphere, a currently understudied Earth environment. Microorganisms in the Stratosphere (MIST), a NASA KSC project, is attempting to characterize the diversity of microbes that exist at stratospheric altitudes from approximately 10 km to 50 km.

Smith (2013)¹ describes multiple collection methods that can be used in aerobiology research. These include precipitation, aircraft, remote sensing, rockets, summit stations, and balloons. Balloons are attractive because of their potential to remain at altitude for weeks at a time, which allows for greater collection volume¹.

One of the major concerns with aerobiology studies is contamination of sampling devices. It has been a constant challenge to distinguish between ground contaminants and collected microbes¹. Smith (2013)¹ suggested that in-flight sterilization, in addition to pre-flight sterilization, may be an effective measure for ensuring the purity of samples collected at altitude. Ultraviolet (UV) irradiation (400 nm to 10 nm wavelengths) is a powerful germicidal method that can be used for in-flight sterilization of a balloon collection unit. Specifically, UVC (280 nm to 100 nm) is the most effective germicidal UV radiation, especially at approximately 254 nm, which is the peak

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absorbance wavelength of many microbes. The radiation kills cells primarily through the destruction of DNA, which prevents replication.

MIST plans on using a meteorological balloon (payload masses of about 4.5 kg) with an attached collection device rather than a large scientific balloon with capacity for 450 kg payloads. To minimize contamination, the collection device will be outfitted with UVC light emitting diodes (LEDs) that will be used to sterilize the collection area during ascent of the balloon. The commercially available UVC LEDs are a state-of-the-art technology. Until recently, only LEDs in the less germicidal UVA and UVB wavelengths were available. Prior to incorporating the UVC LEDs, it is necessary to determine the optimal spatial arrangement of the lights to maximize sterilization within the 30 min to 60 min time constraint of the ascent. Determinations of the optimal angle and distance relative to the target in the shortest time period will allow for both highly effective sterilization and knowledge of margins for engineering constraints of the device.

Bacillus pumilus SAFR-032 endospores will be used as the challenge organism for measuring viable germination reduction after UVC exposure at different times, angles, and distances. Bacterial endospores in general have high radiation tolerance compared to vegetative cells. This particular isolate of *B. pumilus* spores have especially high radiation resistance². High reduction values against these spores would be indicative of more complete UVC germicidal action against less hardy organisms that are likely to contaminate the area.

II. Materials and Methods

A. Spore Harvest, Purification, and Dilution

A *B. pumilus* SAFR-032 spore stock was initially germinated in Difco nutrient broth overnight (100 μ L of 10^7 spores/mL into each of two flasks with 250 mL of broth, grown for 16 hours at 35°C shaking at 140 rpm) to acquire vegetative cells to start a fresh batch of spores. The germinated, vegetative cells were transferred into base sporulation media (0.5 mL culture into 500 mL media) containing Difco nutrient powder, potassium chloride, magnesium sulfate heptahydrate, calcium chloride, manganese chloride, and ferrous sulfate. The inoculated media was incubated at 35°C shaking at 140 rpm for approximately 100 hours.

Spores were purified based on a Nicholson and Setlow (1990) protocol³. The water washing method was adapted to minimize time of purification. The culture was divided into 50 mL Falcon tubes (42 mL into each). The tubes were then centrifuged at maximum speed for 20 min. The supernatant was removed and pellets were re-suspended in 10 mL of cold sterile DI water. Each tube underwent three washes with 10 mL sterile DI water, centrifuged at maximum speed for 20 min. After the initial set of washes, samples were stored in 8 mL DI water at 4°C overnight. Each tube with spores underwent a further two washes (one each subsequent day) in 10 mL DI water, centrifuging at maximum speed for 40 min. Spores were stored each night in 8 mL DI water at 4°C. After the second 40 min wash, spores were re-suspended in 0.5 mL sterile DI water and transferred to a single glass tube that was

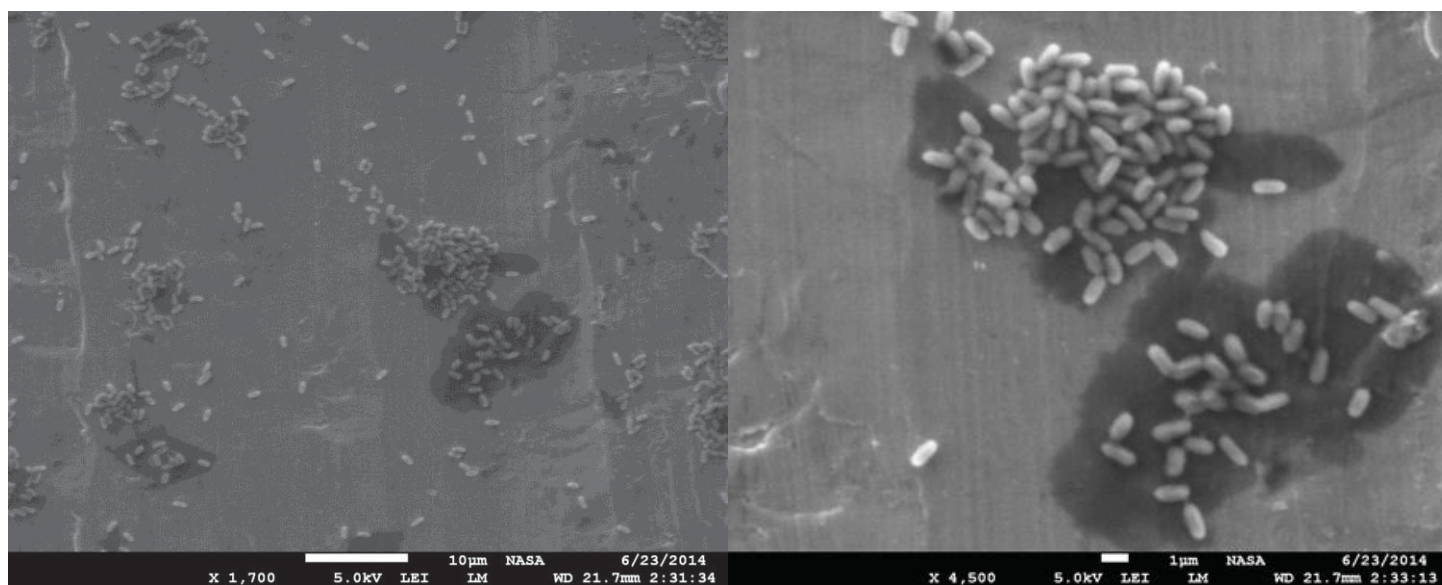


Figure 1. Left: SEM image of typical spore sample at 1700x magnification. Note that some clumping does occur, but the spores are not contaminated with any large, vegetative cells. Right: Close-up of spores with the SEM at 4500x magnification. Note the scale of 1 μ m relative to each spore. Spores are expected to be approximately 1-1.5 μ m.

stored at 4°C.

Spore purity was measured with a scanning electron microscope. Images were visually evaluated and spores were observed based on size and ovoid shape. It was determined that the spore purity was very high (>99%) with no visible vegetative or sporulating cells (see Figure 1).

Spore density was estimated using colony counting on solid nutrient plates and Most Probable Number analysis (discussed below). It was estimated that the spore stock suspension contained approximately 5.61×10^9 spores per mL. With this concentration known, the stock was diluted in sterile DI water to a concentration of approximately 1×10^5 spores per mL. From this dilution, 100 μ L were doped onto aluminum coupons (1.7 cm x 5.4 cm), resulting in about 10,000 spores being present on each coupon to be tested. Coupons were allowed to air dry overnight and were stored in sterile boxes (see Figure 2).



Figure 2. Sterilized empty pipette tip boxes were used to store the metal coupons as shown above. Each coupon was marked with a small purple dot to indicate the side with the spores.

B. UVC Test Stand, Protective Case, LED, and Power Source

A test stand was developed with MIST lead engineer, Michael Lane. Key components of the test stand include the ability to set the distance and angle of the UV LED relative to the inoculated coupons and to mount the coupons underneath a spot designed to hold the LED (see Figure 3). The bridge that holds the LED is able to move vertically and be lined up with markings in half centimeter intervals from 1 cm to 5 cm. The bridge can also be angled between 45° and 90° as indicated by markings at 15° intervals. An external power source connects to the LED through the prongs on top of the bridge. One LED can be used at a time, but up to three coupons can be mounted to determine the spreading of the effective germicidal light (beyond the scope of this present work).

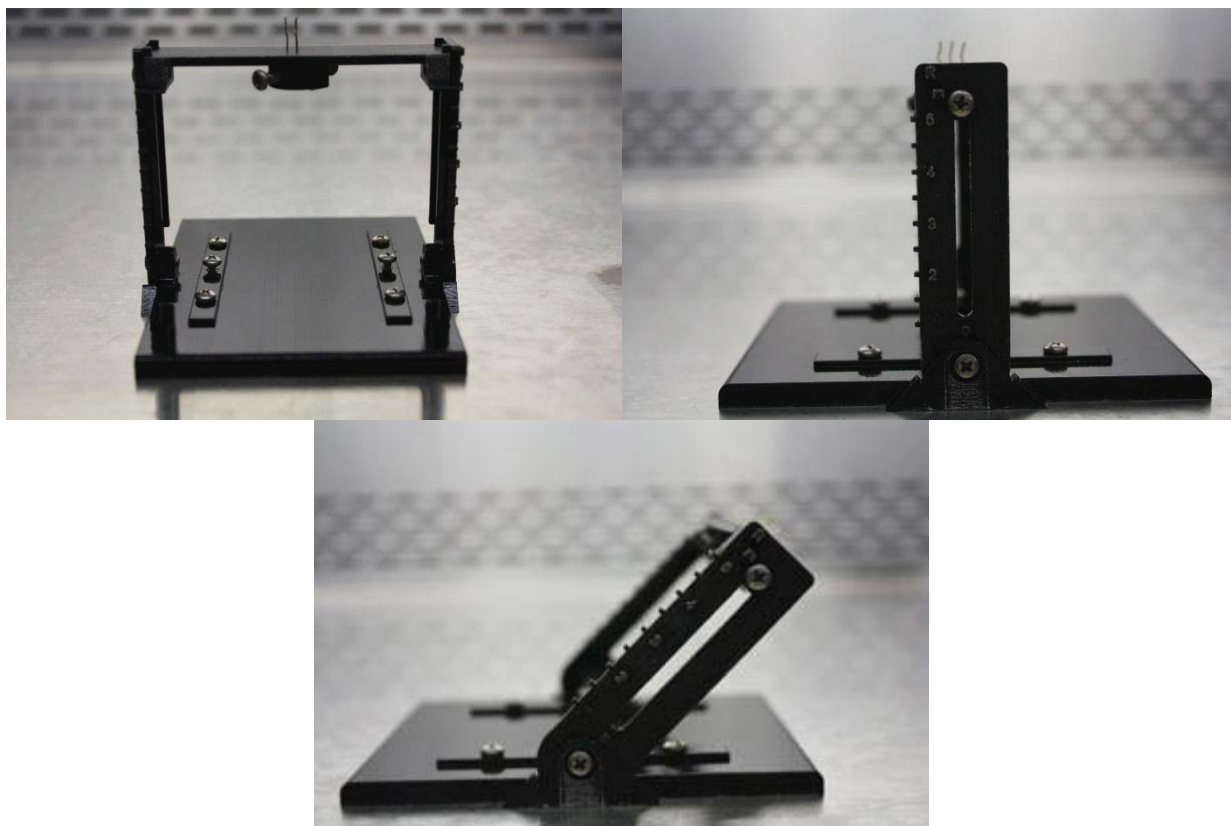


Figure 3. Three views of the UV test stand that was used for experiments. Top left: front view showing screws for coupon attachment and LED placement. Top right: side view showing the different angles and distances possible to test. Bottom: side view demonstrating change in angle.

Because of the inherent dangers of working with ultraviolet radiation, a protective enclosure was made to block the UVC. This was made from a commercially available 2.0 gallon black nursery planter. Incisions were made to accommodate the wire of the power source. All openings were covered with commercially available plumber's putty to further prevent UV leakage. All testing occurred behind a UV-proof glass window in a biological safety cabinet (Labgard Class II, Type A/B3, Model NU-600 Series 24, NuAire, Inc.) as extra precaution.

A UVC LED from QPhotonics, L.L.C. (Part Number: UVTOP270TO39FW) was used for all experiments. This LED had a maximum voltage of 5.668 V, a maximum current set to 20.00 mA, a maximum power output of 1160 μ W, peak wavelength of 271.1 nm, and spectral width of 10.3 nm. A Qphotonics AC/DC power supply with current control was used to power the LED. The power supply was set to 20.0 mA at the start of each test. The current dropped to as low as 18.9 mA over the course of each test (generally dropping more after longer test periods). Most of the tests did not drop below 19.4 mA.

C. Spatial Arrangement Testing

Three variables were tested: time, distance, and angle. Every individual test was performed in triplicate in randomized order within the variable and randomized doped coupons. Refer to Figure 4 for experimental setup.

Time was the first variable tested to determine an ideal time to experiment with different angles and distances. All time tests occurred at 5 cm and 90° relative to the coupon. Times from 5 min to 25 min were tested in 5 min intervals. The LED was turned to a current of 20 mA, which would slowly decrease (minimum value of 18.9 mA), largely as a function of time.

Variation in distances of the LED to the coupon was explored next. These tests occurred at 90° for 15 min each. Distances from 1 cm to 5 cm were tested in 1 cm intervals in triplicate.

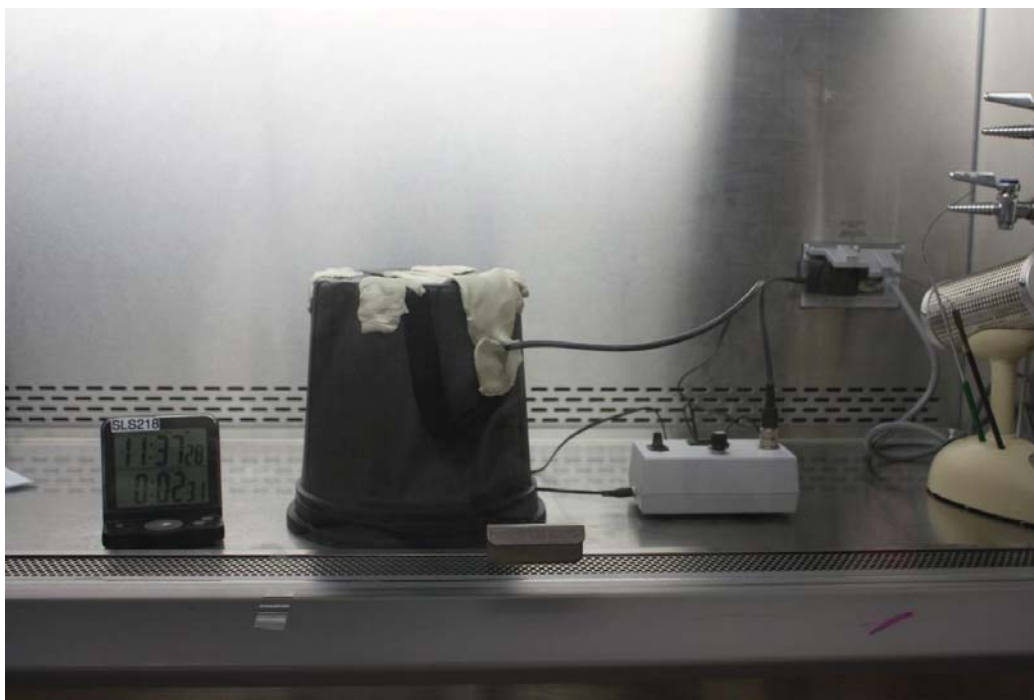


Figure 4. Experimental setup. The power supply wire would enter through the openings of the protective case. Remaining open areas would be covered with plumber's putty to ensure safety. The UV test stand is connected to the wire. This setup is behind a glass window in a biological safety cabinet.

Angle, the final variable, was tested next. Tests were performed with the LED at 5 cm from the coupon with 15 min of irradiation. The angle was changed for each test from 45° to 90° in 15° increments. Each angle was tested in triplicate.

D. Viable Spore Reduction Analysis

The Most Probable Number (MPN) cell enumeration method was employed to measure relative reductions in spore viability across the different treatments. MPN is a statistical method described by Halvorson and Ziegler (1933)⁴ and further refined by others^{2,5}. Briefly, MPN uses a series of dilutions in a liquid spore medium to

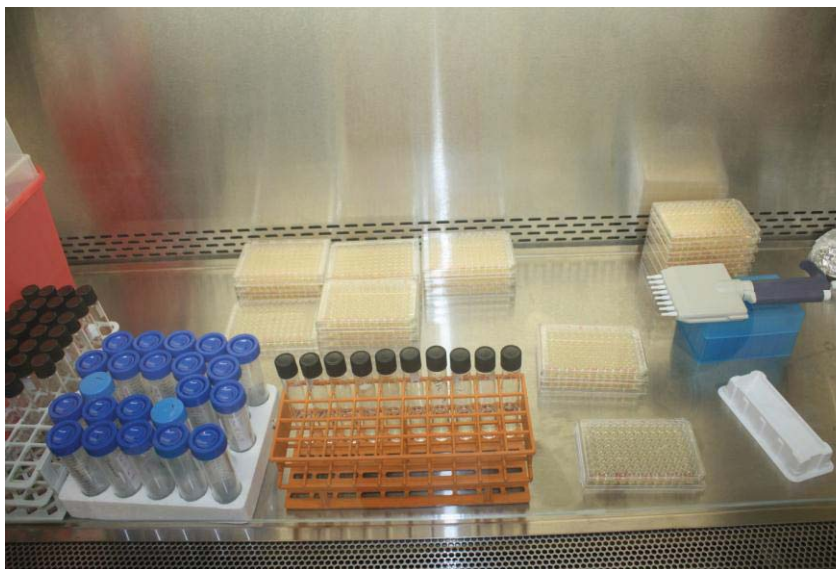


Figure 5. MPN setup. Blue Falcon tubes contained the treated spore coupons. The rack was stocked with test tubes that were filled with 9 mL of sterile DI water for the serial dilutions. The 96-well plates were labeled and filled with MPN spore media. The spores were diluted in and each dilution was placed in 16 wells of the plate with a multi-channel pipette with well boats such as the one on the right of the image.

determine the dilution of attenuation of growth. Through a series of equations, a set of three wells with positive growth in a 96-well plate will yield a number that represents the number of viable cells in the stock when multiplied by the dilution factor of the third dilution in the set. The stock is defined as the treated inoculated coupon that has had its spores knocked off into 10 mL sterile DI water by vortexing with sterile glass beads. The spore media consisted of DI water, Difco nutrient powder, potassium chloride, calcium chloride, ferric chloride, manganese sulfate, magnesium sulfate, and D-glucose. Refer to Figure 5 for an image of MPN plate preparation and Figure 6 for a labeled diagram of a MPN plate. It is expected that as more spores are killed by the UV, growth will attenuate closer to the left side of the well plate up to negative growth in all wells.

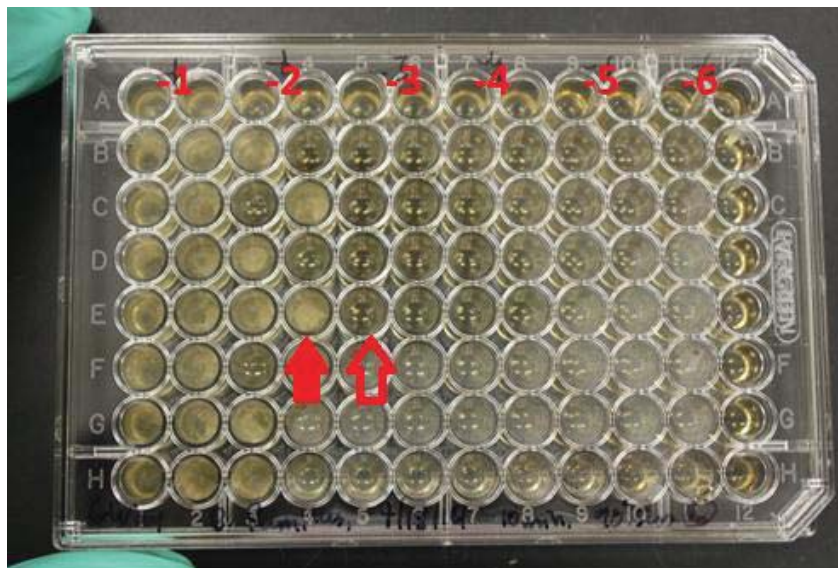


Figure 6. Typical results of a MPN plate. Top numbers indicate the dilution factor (10^x). Each dilution factor included two columns of wells for a total of 16 wells each. The filled red arrow indicates a well with positive growth. The unfilled arrow points to a well without growth.

E. Analysis

All data analysis was performed using Microsoft Excel 2010. Student's *t*-tests were calculated assuming equal variance among samples. Differences were considered significant for *p*-values less than 0.05.

III. Results

A. Controls

Three control coupons were used to determine a baseline number for starting viable spores for comparison to the UV-treated coupons. The controls were not exposed to the UVC LED. The average MPN for the three control coupons was 640 viable spores per mL of the “stock” that contained the coupon and 10 mL sterile DI water.

B. Time Series

The time series tests resulted in an indication of exponential decay of the most probable number of viable spores over time (see Figure 7). A significant reduction in viable spores after UV treatment compared to the control was not observed until 15 min of exposure. After this level of UV exposure, the average most probable number of viable spores was 162 compared to approximately 640 for the control. Increased times of exposure at 20 min and 25 min resulted in further modest decreases in spore viability, but a significant decrease relative to 15 min was not observed. From this information, it was determined that 15 min would be an effective time for testing both distance and angle. This time was chosen because of its notable difference relative to the control to distinguish from background noise without necessarily overexposing the spores, increasing the likelihood of acquiring more informative reduction curves.

Average Most Probable Numbers of Viable *B. pumilus* Spores for Different Times of UVC Exposure at 5 cm and 90° Angle of Incidence

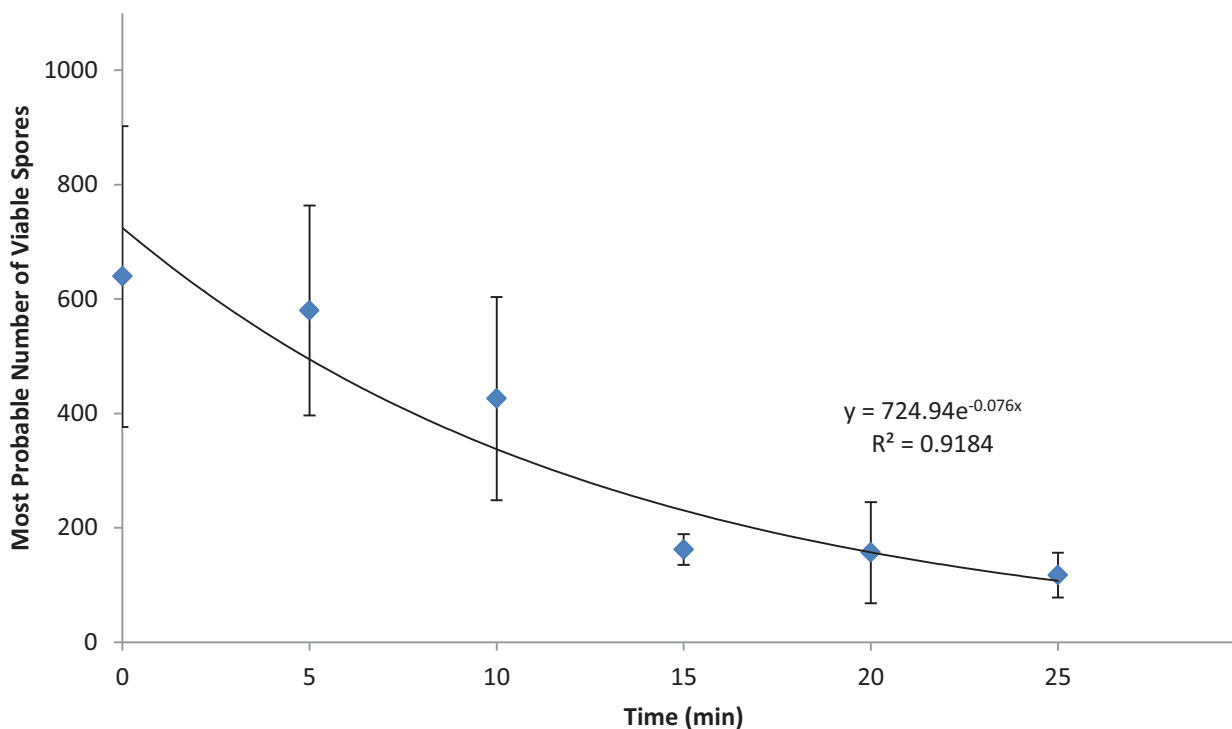


Figure 7. Plot of MPN vs. Time in min. At 15 min and longer, there were significant decreases in viable spores relative to the control. Error bars represent ± 1 standard deviation.

C. Distance

Decreasing the distance of the incident UV radiation resulted in a similar exponential decrease (see Figure 8) in viable spores as increasing time. The viable spore reduction was highly significantly greater at 4 cm compared to 5 cm ($p=0.0008$), with most probable number averages of 31.67 and 162, respectively. Further, reductions at 2 cm and 1 cm were significantly less than that at 4 cm ($p=0.0131$ and 0.0109 , respectively).

**Average Most Probable Numbers of Viable *B. pumilus* Spores
for Different Distances of UVC Exposure for 15 min and 90°
Angle of Incidence**

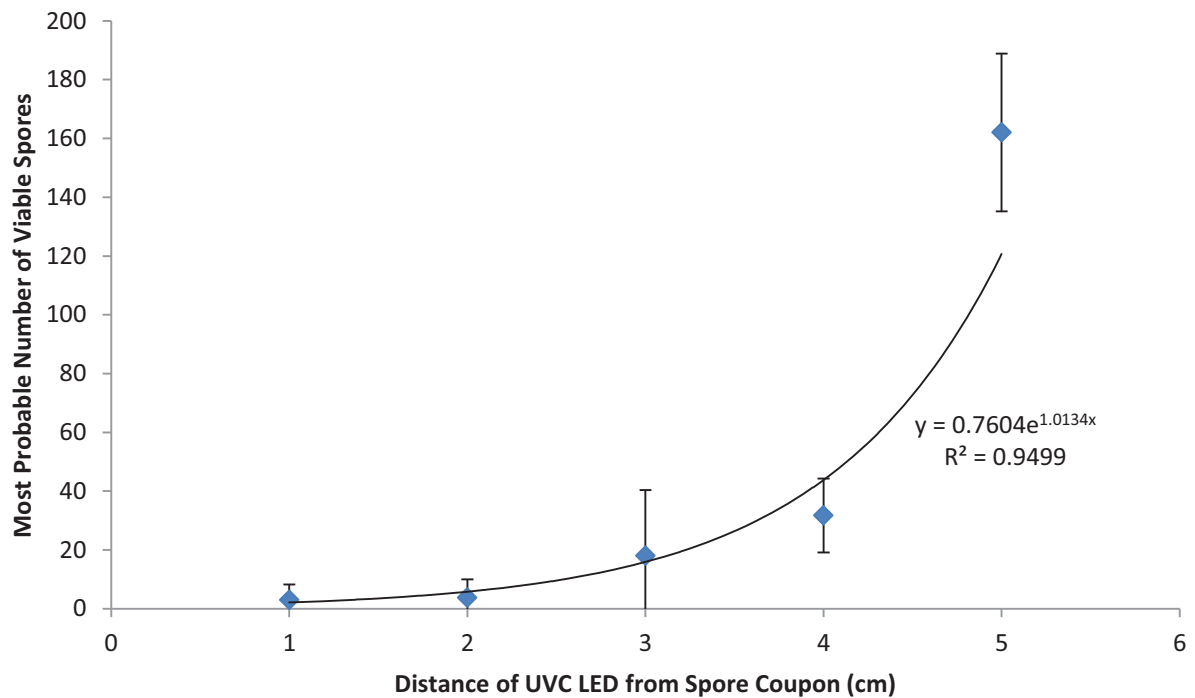


Figure 8. Plot of MPN vs. Distance from the UV LED in centimeters. Decreasing the distance from 5 cm significantly lowered the number of viable spores. Error bars represent ± 1 standard deviation.

Average Most Probable Numbers of Viable *B. pumilus* Spores for Different Angles of UVC Exposure at 5 cm and for 15 min

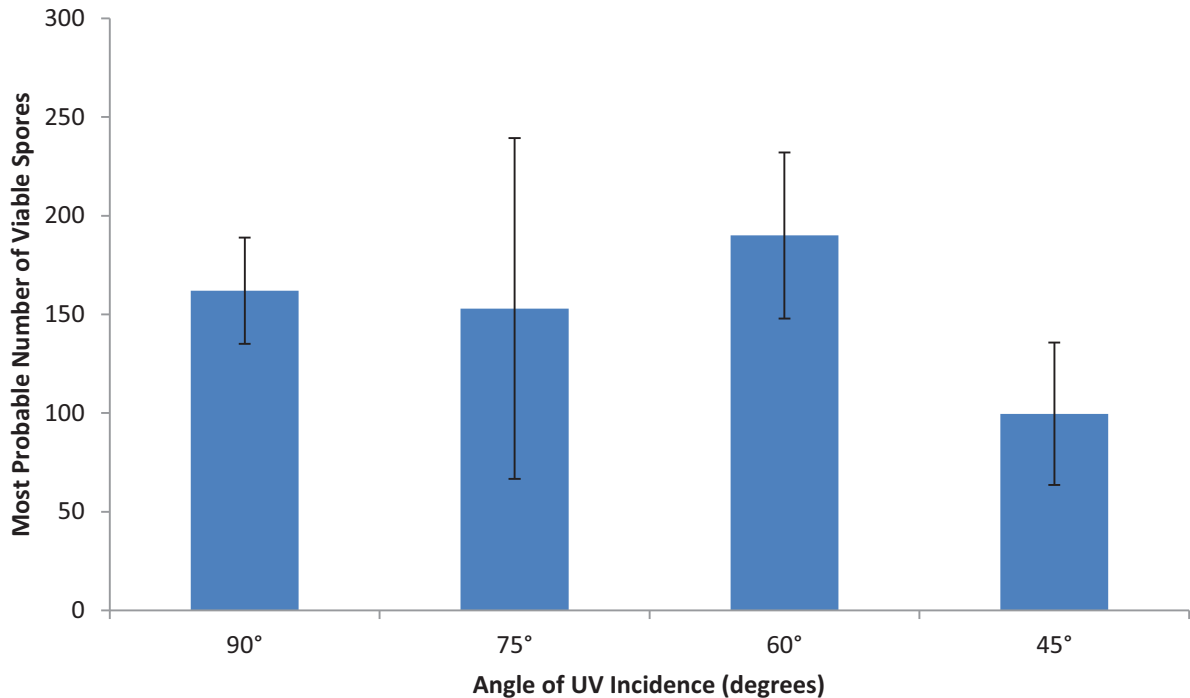


Figure 9. Bar graph of MPN vs. Angle of UV. Overall, only one significant difference was observed and no trends were determined. The 45° angle had significantly lower viable spore counts than at 60°, but no other significant differences were found. Error bars represent ± 1 standard deviation.

D. Angle

Four different angles were tested at a distance of 5 cm for 15 min each (see Figure 9). For the most part, there were no significant differences between any of the angles. The one exception is the difference between 60° and 45°. The student's t-test for these variables indicated a p -value of 0.0477. No clear trends were determined from these data.

IV. Discussion

The data suggest that time and distance are the most important variables in terms of viable spore reduction. For most effectively sterilizing the balloon collection device prototype, it would be optimal to arrange the lights within 4 cm. The data also suggest that the angle of incidence of the UV has no significant effects on spore reduction, so there is greater potential for where the lights may be placed within the 4 cm. Closer distances and longer times will be more effective. Similarly, when the collection device is ascending, it is important to have the device exposed to the sterilizing UV LEDs for a minimum of 15 minutes. Increasing the time will continue to increase the chances of killing all surviving microbes.

It is important to note that this was a relatively conservative study in which especially UV resistant spores were used as a challenge organism. Most organisms that would contaminate the surface of the device would be killed with even less exposure to the radiation. Additionally, the coupons were doped with a much higher number of spores than would be expected on the hardware. Furthermore, only a single LED, which was relatively weak in its power output, was used for these tests. Multiple LEDs with greater power outputs will be used for sterilizing the collection device. Using 2 or 3 LEDs should vastly increase the likelihood of complete sterilization even in with high concentrations of present microbes.

Future studies should continue determining the optimal arrangement required to achieve effectively 100% viable spore reductions by varying the times and distances of UV exposure. Upon optimizing these reduction curves, it would be necessary to verify similar results with the stronger LEDs working simultaneously to sterilize the surface of the collection device. A systematic approach based on the results presented here would allow for a relatively quick confirmation of high spore kill rates. After initial *in-situ* testing during flight, this work could be applied to a number of planetary protection endeavors to minimize the possibility of forward contamination. This would be especially useful for robotic instruments/parts that come in direct contact with other planetary atmospheres and surfaces when testing for biosignatures on planetary bodies such as the upper atmosphere of Venus, surface of Mars, oceans of Titan, and ice/ocean of Europa.

Acknowledgments

G. M. Wong thanks David J. Smith, Ph.D. for providing the opportunity to work on MIST, his excellent guidance through the process, and contagious enthusiasm for astrobiology. G.M. Wong thanks Mike Lane for his work in developing and building the UV test stand. G.M. Wong thanks Christina Khodadad, Ph.D., Mary Hummerick, and Andrew Schuerger, Ph.D. for their laboratory support/advice as well as the Advanced Life Support Laboratory and Environmental Microbiology groups at KSC. G.M. Wong also thanks the University Space Research Association for funding and NASA Kennedy Space Center for its facilities.

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